High-level expression of human c-Src can cause a spherical morphology without loss of anchorage-dependent growth of NIH 3T3 cells

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Abstract To investigate whether overexpression of human c-Src leads to cell rounding in anchorage-dependent NIH 3T3 fibroblasts, we have established c-Src-inducible cell lines using a *lac* repressor–operator system. RN1 cells, which expressed c-Src at a high level after induction, exhibited a spherical morphology and ceased to grow in monolayer culture. RN1 cells, however, exhibited neither focus-forming ability nor anchorage-independent growth potential with or without induction. Induced RN1 c-Src was phosphorylated at Ser^{75}, a previously reported spherical cell-associated site, and at Tyr^{419}. These data demonstrated for the first time that highly elevated human c-Src tyrosine kinase activity can cause NIH 3T3 cells to have a spherical morphology without loss of anchorage-dependent growth. The inducible cell line should be useful to study the mechanism for cell rounding by c-Src.

Key words: Human c-Src; Inducible expression; NIH 3T3 fibroblast; Cell rounding; Anchorage-dependent growth; Ser^{75} phosphorylation

1. Introduction

The cellular c-*src* gene product, c-Src, is a membrane-associated 60 kDa tyrosine kinase, which is structurally homologous to the transforming product, v-Src. The normal cellular function of c-Src is not fully understood, although v-Src deregulates cell growth [1,2]. High-level expression of c-Src in terminally differentiated cells such as neurons [3] and platelets [4], suggests that its cellular activities are unrelated to growth regulation.

Recent studies suggest that c-Src is involved in regulation of cell morphology [5] and cell adhesion to a substratum [6]. c-Src is activated during fibroblast mitosis accompanied by phosphorylation of serine and threonine in the amino terminal regulatory domain of the protein [7,8]. The mitotic cells decrease the cell adhesion to a substratum and then round up. We have previously found that a Ser^{75} phosphorylation event in human c-Src correlates with cell spherical morphology [9]. Human retinoblastoma Y79 cells, growing in a suspension of spherical cells, express c-Src phosphorylated on Ser^{75} throughout the cell cycle. c-Src from fibroblast-like and epithelial-like cell lines adherent to substrata is phosphorylated on Ser^{75} only during mitosis. Furthermore, v-*src*-transformed chicken and rat embryo fibroblasts display a spherical and refractile morphology similar to mitotic cells [10], implying that these v-*src*-induced effects are caused by deregulation of the putative normal function of cell rounding in c-Src.

To test whether human c-Src plays a role in cell rounding, we overexpressed human c-Src in NIH 3T3 fibroblasts. We induced the expression of human c-Src in NIH 3T3 fibroblasts using the *lac* repressor and operator system. Induction of high-level expression of c-Src with isopropyl-β-D-thiogalactoside (IPTG) changed spread and adherent fibroblastic cells into spherical cells and inhibited cell growth. We found that these changes were dependent on high-level activity of human c-Src tyrosine kinase and were accompanied by specific phosphorylation of the Ser^{75} in human c-Src.

2. Materials and methods

2.1. Materials

LacSwitch Inducible Mammalian Expression System was purchased from Stratagene. Geneticin (G418) was from Life technologies. Hygromycin B was from Sigma. Monoclonal anti-Src antibody 327 was obtained from Oncogene Science. Monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology. Herbimycin A was purchased from WAKO, Japan.

2.2. Cells

NIH 3T3 cells were obtained from the Health Science Research Resources Bank (Osaka) and cultured in DMEM plus 10% calf serum at 37°C.

2.3. Transfection

A human c-*src* cDNA [9] was cloned into pOPI3 operator vector (Stratagene). NIH 3T3 cells (8×10^6) were mixed on ice with 25 μg of p3′SS *lac*-repressor-expressing vector (Stratagene) and 30 μg of pOPI3-*src*, and then electroporated with a Bio-Rad Gene Pulser at a setting of 1.2 kV and 25 μF. After 48 h, cells were cultured in selective medium containing hygromycin B (200 μg/ml) and G418 (500 μg/ml). After 21–30 days, resistant colonies were selected and expanded in selective medium. The stably transfected cells were induced by the addition of IPTG (0.1–5 mM) to the media.

2.4. Immunoprecipitation

Cells were lysed with detergent lysis buffer [11] supplemented with 50 mM β-glycerophosphate, 25 mM NaF and 0.15 mM sodium orthovanadate. c-Src was immunoprecipitated with anti-Src antibody from cell lysates and separated on SDS-10% PAGE as described [11].

2.5. Immunoblot analysis

c-Src levels were measured by immunoblot analysis as described previously [11] except that colored bands were detected with a 420OE Scanner (Pharmacia). For tyrosine phosphorylation analysis of cellular proteins, lysate proteins (20 μg) were separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with a 1:4000 dilution of anti-phosphotyrosine antibody. The filters were treated with a 1:1000 dilution of biotinylated goat anti-mouse IgG (Life Technologies) and then with a 1:1000 dilution of tangerine-labeled streptoavidin (Molecular Probes). Phosphorylated proteins were detected with an FMBIO-100 Fluorescent Image Analyzer (TAKARA).

2.6. Kinase assays

Autophosphorylation and enolase phosphorylation were measured by immune complex kinase assay and detected by BAS2000 (Fuji) as described previously [11].

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2.7. Radiolabeling of cells

RN1 cells were cultured with or without IPTG treatment for 18 h and were labeled with 1 mCi/ml [^{32}P]orthophosphate (ICN) as described previously [9] except that IPTG was included in the media for the induced cells.

2.8. Phosphopeptide analyses

V8 protease and 2-dimensional tryptic phosphopeptide mappings were performed as described previously [11]. Cyanogen bromide digests [12] were analyzed on Tricine-SDS-PAGE (16.5% T/3% C) as described [13]. Phosphoamino acid analysis was conducted as previously described [11].

3. Results

3.1. Human c-Src overexpression induces a spherical morphology of NIH 3T3 cells

We sought to test whether overexpression of human c-*src* causes anchorage-dependent fibroblasts to have a spherical morphology. Fibroblasts require adhesion to substrata for cell growth and survival [14]. If they are held detached and rounded up, they cease growing. Accordingly, constitutive overexpression of a gene enhancing cell rounding will inhibit cell growth and survival, unless it induces transformation of cells to exhibit anchorage independence of growth. Overexpression of chicken and human c-*src* does not induce the transformation of mouse NIH 3T3 fibroblasts [15–17]. Therefore, we have stably co-transfected NIH 3T3 cells with a *lac* operator vector containing human c-*src* cDNA and a *lac* repressor-expressing vector to obtain c-Src-inducible cell lines.

Immunoblotting analysis showed that isolated clonal cell lines expressed various elevated levels of c-Src after induction for 20 h with IPTG (Fig. 1A). The relative levels of c-Src from RN1, RN2, RN3 and RN4 cell lines after induction were 63-, 37-, 33-, 12-fold, respectively, of that of the parent NIH 3T3 cells. In the parent NIH 3T3 cells and control vector transfectants, IPTG did not induce the elevation of c-Src levels (Fig. 1A). All four human c-Src-expressing cell lines produced kinase-active c-Src, as judged from autophosphorylation assay of the immunoprecipitated c-Src (Fig. 1A). Spherical cells, part of which are detached from the substrata, appeared in RN1 cells expressing the highest level of c-Src after IPTG induction (Fig. 2A,B). A similar morphological change was never observed in cell lines (RN2-RN4) expressing lower levels of c-Src than RN1, or in the parent NIH 3T3 cells (Fig. 2G,H).

3.2. Correlation between c-Src kinase activity level and cell rounding in RN1 cells

To study the relationship of both c-Src and its kinase activity level to morphological alteration in RN1 cells, we performed immunoblotting and in vitro immune complex kinase assay of the immunoprecipitated c-Src from these cells during induction for 24 h with IPTG. Both levels of c-Src and its kinase activity increased 5- and 50-fold, respectively, by 24 h after initiation of the induction (Fig. 1B). c-Src levels were nearly equal after induction for 20–72 h (data not shown). A small number of spherical cells appeared after 10 h induction, which produced more than 70% of the level of c-Src after 24 h induction (Fig. 1B). The percentage of spherical cells increased to about 60% of the total cells after 24 h induction. Furthermore, 48 h after IPTG removal, the levels of c-Src and its kinase activity decreased to about half the levels of the continuously induced cells (Fig. 1B), and concomitantly, spherical cells adhered to the substratum and spread to restore

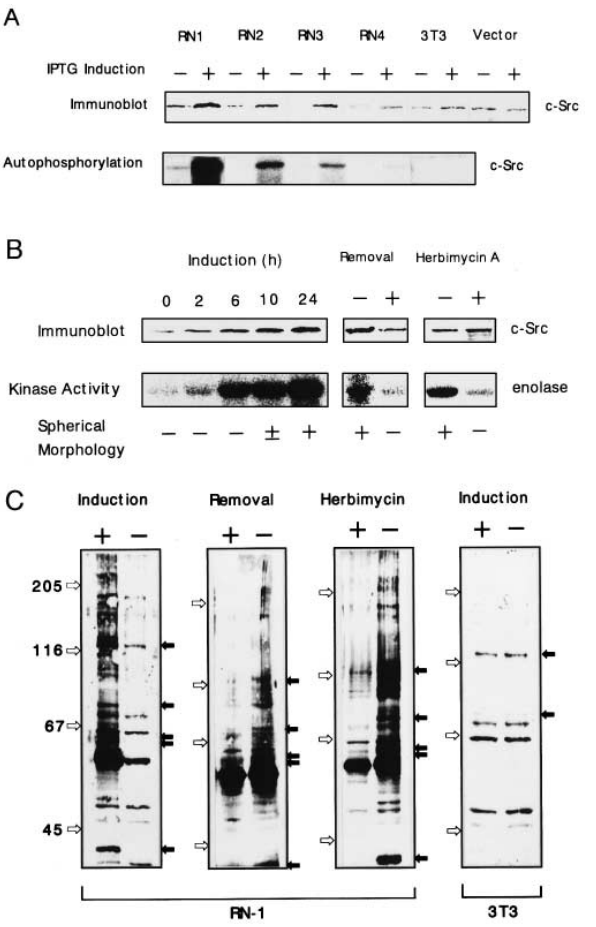

Fig. 1. (A) c-Src expression in transfected NIH 3T3 cells. Human c-Src was expressed in NIH 3T3 cells using the LacSwitch Inducible Mammalian Expression System. Cells were co-transfected with a p3′SS repressor vector and a pOPI3-*src* vector, and clones resistant to G418 and hygromycin B were isolated. The clonal cells at the middle log phase were treated or untreated with 0.5 mM IPTG for 20 h and lysed. c-Src was immunoprecipitated from the cell lysates (RN1-4 cells: 10 μg of lysate protein; parent 3T3 cells and control vector transfectants: 50 μg of lysate protein). The immunoprecipitates were subjected to anti-Src immunoblot analysis and autophosphorylation assay, respectively. c-Src expression level and rate of the spherical cells were unchanged in the range between 0.1 and 5 mM IPTG. (B) c-Src expression level and morphology in RN1 cells. (Induction) RN1 cells were induced with IPTG for the indicated times as described above. (Removal) RN1 cells induced for 24 h with IPTG were washed with PBS exhaustively and then cultured for 48 h in the fresh medium from which IPTG was removed (+) or not (−). (Herbimycin A) RN1 cells induced for 6 h were treated with 0.05 μg/ml herbimycin A (+) or DMSO containing no herbimycin A (−) and then cultured continuously for 18 h with the inducer included. These cells were lysed, and the immunoprecipitated c-Src per 10 μg of lysate protein was subjected to anti-Src immunoblot analysis and enolase phosphorylation assay, respectively. (C) Tyrosine phosphorylation of cellular proteins in RN1 cells. Lysates were prepared from cells treated (+) or untreated (−) with IPTG for 24 h (induction), removal of IPTG (removal), or herbimycin A (herbimycin) as described above. Lysate proteins were separated by SDS-PAGE and then subjected to anti-phosphotyrosine immunoblot analysis. White arrows show the position of molecular mass standards 205, 116, 67, and 45 kDa. Black arrows show *Rf* values corresponding to molecular masses of 118, 77, 66, 63, and 42 kDa.

the fibroblastic morphology (Fig. 2C). In contrast, the continued induction for 48 h retained the spherical morphology (Fig. 2D).

Next, we tested whether a Src kinase specific inhibitor, herbimycin A [18], inhibits the change in RN1 cells. When herbimycin A was added to the culture after 6 h induction to a final concentration of 0.05 μg/ml, the level of c-Src kinase activity decreased to the one-third of that of the control culture after further induction for 18 h. Herbimycin A did not change the c-Src level at this concentration (Fig. 1B). Spherical cells were about 3-fold less than those in the culture without herbimycin A (Fig. 2E,F).

Finally, we examined the pattern of tyrosine phosphorylation of cellular proteins by immunoblotting the cell lysates with anti-phosphotyrosine antibody. Tyrosine phosphorylations of 118, 77, 66, 63 and 42 kDa proteins in RN1 cells

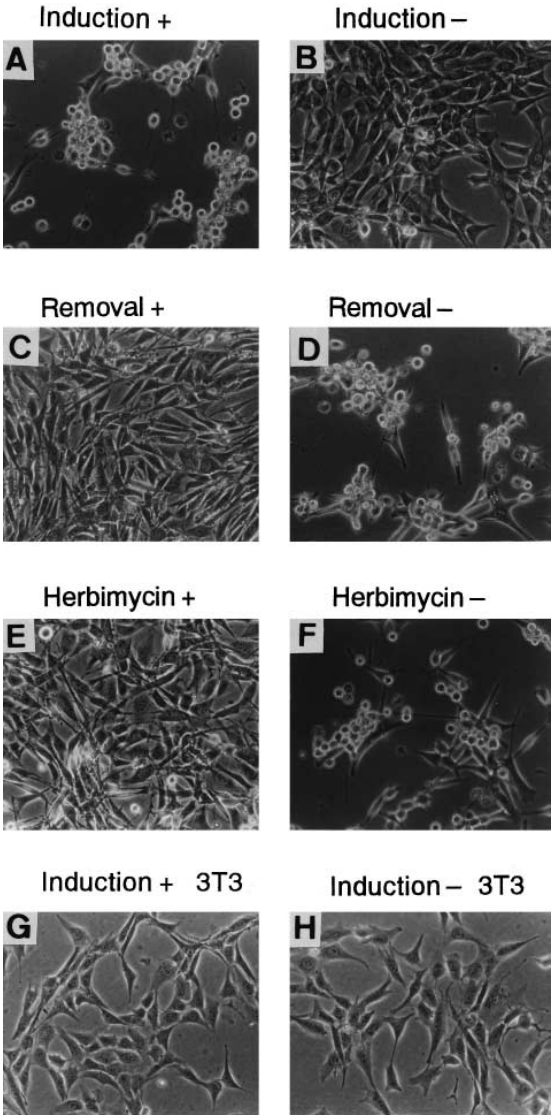

Fig. 2. Morphologies of RN1 cells with or without IPTG induction. RN1 cells (A and B) and parent NIH 3T3 cells (G and H) at the middle log phases were treated (A and G) or untreated (B and H) with IPTG for 24 h. The treated RN1 cells were washed and then cultured for 48 h with (C) or without (D) removal of IPTG as described in Fig. 1B. RN1 cells were treated (E) or untreated (F) with herbimycin A as described in Fig. 1B. Magnification: ×117.

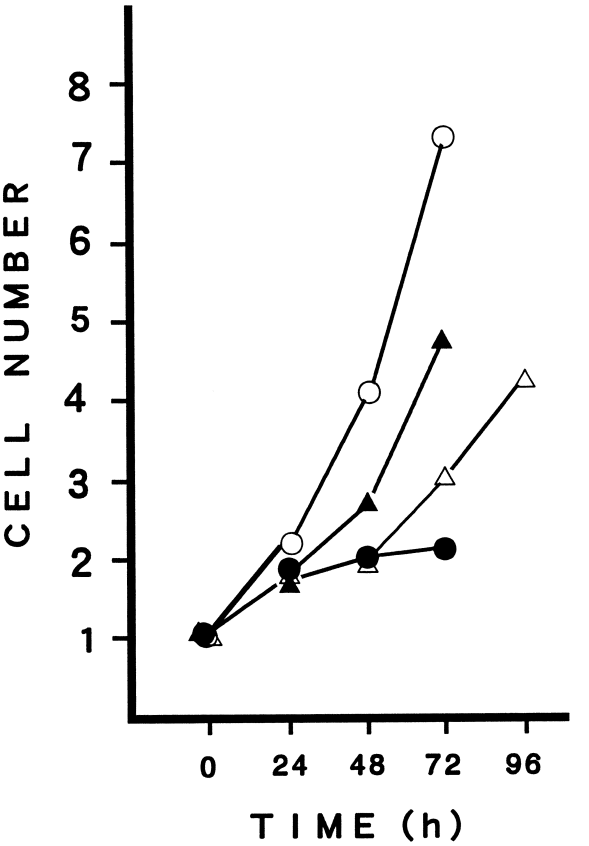

Fig. 3. Effects of IPTG induction on cell growth of RN1 cells. RN1 cells (2×10^5 cells) were seeded per 6 cm culture dish and cultured for 2 days. The cultures were induced (●), uninduced (○), induced for 24 h followed by removal of IPTG (△), or induced for 6 h followed by herbimycin A treatment as described in Fig. 1B (▲). The attached and detached cells combined were counted in duplicate dishes at the indicated times. Cell numbers were normalized to the number of cells at the start of IPTG treatment (zero time point, assigned a value of 1).

increased more than 4-fold after induction, suggesting that the five proteins were phosphorylated directly or indirectly by c-Src kinase (Fig. 1C). Moreover, these tyrosine phosphorylations were all reduced more than 3-fold and 6-fold, respectively, by IPTG removal and herbimycin A treatment (Fig. 1C). No change was detected in the parent 3T3 cells after induction (Fig. 1C).

3.3. *Effects of overexpressed c-Src on RN1 proliferation*

To test whether RN1 cells after induction of human c-Src retain an anchorage dependence of growth in monolayer culture such as parent 3T3 fibroblasts, we assayed the effects of IPTG induction on cell growth of RN1 cells (Fig. 3). The IPTG induction led to inhibition of RN1-cell proliferation incidental to cell rounding (Fig. 2AFig. 3). The removal of IPTG and the herbimycin A treatment restored RN1-cell proliferation maintaining a fibroblastic morphology (Fig. 2C,EFig. 3). Thus, c-Src-overexpressing RN1 cells exhibiting decreased adhesion and a spherical morphology failed to increase in cell number. RN1 cells in suspension, irrespective of IPTG treatment, exhibited no colony formation in soft agar and no growth potential in agar-coated dishes [14] (data not shown). These results indicate that the RN1 cells have anchorage dependence.

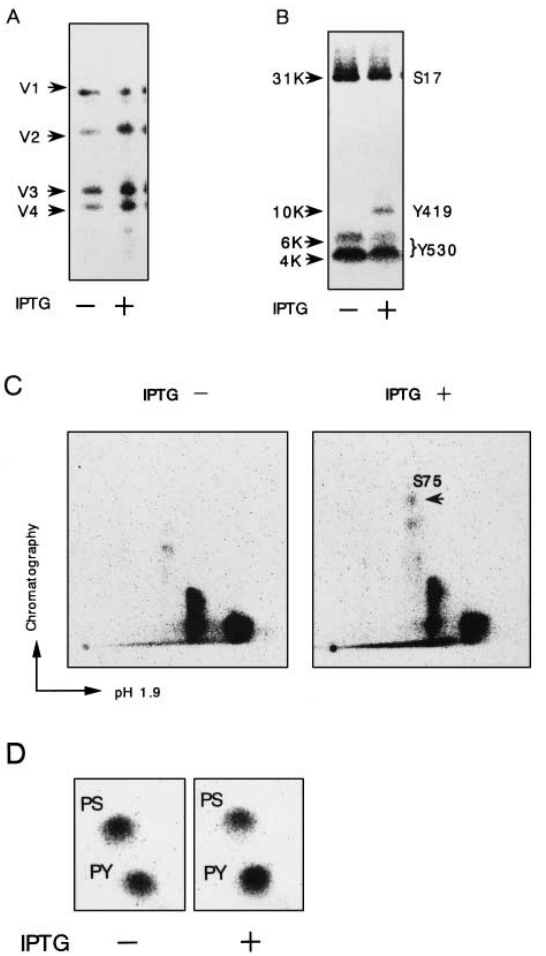

Fig. 4. Phosphorylation status of RN1 cells with or without IPTG treatment. A: V8 peptide maps of RN1 c-Src. B: Cyanogen bromide maps of RN1 c-Src. C: Two-dimensional phosphopeptide maps of RN1 c-Src. D: Phosphoamino acid analysis of RN1 c-Src. RN1 cells treated or untreated with IPTG for 18 h were labeled with [^{32}P]orthophosphate. c-Src was immunoprecipitated, separated on SDS-PAGE, and subjected to V8 protease mapping, to cyanogen bromide cleavage, or to phosphoamino acid analysis. The N-terminal 16 kDa V8 fragments were subjected to 2-dimensional tryptic phosphopeptide analysis. V1, N-terminal 34 kDa fragments; V2, C-terminal 26 kDa fragments; V3 and V4, N-terminal 18 kDa and 16 kDa fragments, respectively, derived from further cleavage of V1. PS, phosphoserine; PY, phosphotyrosine.

3.4. Comparison of phosphorylation status of RN1 c-Src with or without IPTG induction

We examined the alteration of the phosphorylation status of overexpressed c-Src from RN1 cells after induction by phosphopeptide mapping and phosphoamino acid analysis. Patterns of V8 mapping of in vivo phosphorylated RN1 c-Src were unchanged irrespective of IPTG treatment (Fig. 4A). The patterns were identical to that of normal c-Src [11]. The tyrosine phosphorylation status of C-terminal c-Src was analyzed by cyanogen bromide mapping. The level of phosphotyrosine 530, a regulatory site of the kinase activity [19], in induced RN1 cells was the same as that of uninduced RN1 cells, judging from the ratio of the combined radioactivity of 4 and 6 kDa bands containing phosphotyrosine 530 to that of a N-terminal 31 kDa band containing phosphoserine 17 (Fig. 4B). This is consistent with no change in the specific activity of c-Src kinase, which was compared between equal amounts of c-Src from IPTG-treated and untreated RN1 cells (data not shown). On the other hand, phosphotyrosine 419 (10 kDa band) was detected in c-Src only from RN1 cells with IPTG induction (Fig. 4B). To reveal the phosphorylation pattern of N-terminal c-Src, we performed 2-dimensional phosphopeptide mapping of N-terminal V8-digested 16 kDa fragments of RN1 c-Src. Phosphoserine 75-containing peptide was detected in c-Src elevated by IPTG induction (Fig. 4C). The relative intensity of this phosphopeptide [9] in c-Src from RN1 cells after induction was about 4 times that of cells without induction. In phosphoamino acid analysis (Fig. 4D), RN1 c-Src contained phosphoserine and phosphotyrosine, but not phosphothreonine, irrespective of IPTG treatment.

4. Discussion

We examined the effects of overexpressed human c-Src on cell morphology of NIH 3T3 cells using an inducible expression system with a *lac* operator and a *lac* repressor. As shown in Figs. 1–3, RN1 cells induced to express high-level, functional c-Src kinase exhibited a spherical morphology and failed to grow in a monolayer. The time course of induction showed that spherical cells appeared at more than 70% of the maximum expression level of RN1 c-Src. Other cell lines (RN2-4) induced to express less than about 60% level of the RN1 c-Src exhibited no spherical morphology. A decrease to half of the maximum in c-Src level by removal of the inducer restored the fibroblastic morphology and cell growth of RN1 cells. The expression level-dependent behavior of c-Src may reflect the existence of a c-Src kinase level threshold required for the cell rounding. Furthermore, changes in the levels of in vitro c-Src kinase activity after induction are accompanied by those in phosphotyrosine levels of several cellular proteins. Herbimycin A, a Src kinase-specific inhibitor, inhibited the morphological changes in induced RN1 cells, accompanied by a decrease in the in vitro kinase activity and tyrosine phosphorylation of cellular proteins. Thus, these results rule out the possibility that the morphological change was due to a large amount of the protein, and not to the increased level of c-Src kinase activity. Overall, we conclude that the highly elevated level of human c-Src kinase activity can cause spherical cell morphology without loss of anchorage-dependent growth.

RN1 cells with or without induction expressed proteins that exhibited the V8-cleavage pattern of normal human c-Src and not the patterns of activated forms (Fig. 4) such as v-Src [16], mitotic c-Src [7] or neuronal Src [3]. The absence of phosphothreonine suggested that induced RN1 cells have no mitotic, activated c-Src (Fig. 4). Furthermore, cyanogen bromide mapping showed that c-Src activation did not occur through Y530 dephosphorylation after induction. Altogether, these results suggest that the cell rounding in RN1 cells is not ascribed to gross alteration of the primary structure of c-Src, or not to c-Src activation.

In contrast, RN1 cells treated with IPTG produced c-Src phosphorylated additionally on Ser^{75} and Tyr^{419} compared with untreated RN1 cells (Fig. 4). Our previous studies showed that the Ser^{75} phosphorylation event correlates with a spherical morphology in 14 various unsynchronized human tumor cell lines and in mitotic fibroblasts and epithelial-like cells [9]. Thus, Ser^{75} phosphorylation may be involved in cell rounding in RN1 cells. Cdc 2 kinase in vitro phosphorylates

Ser^{72} in chicken c-Src corresponding to Ser^{75} in human c-Src [20]. It is possible that the high-level expression of c-Src in RN1 cells treated with IPTG up-regulates Cdc 2 or other related kinase(s) and then conversely results in phosphorylation of Ser^{75} in overexpressed c-Src. Tyr*419* is an autophosphorylation site, which is not stably phosphorylated in c-Src [21]. High-level expression of c-Src may have stabilized this phosphorylation. The role of this autophosphorylation has been elusive [2,22]. Inducible expression of mutant human c-Src in NIH 3T3 cells will reveal the roles of Ser^{75} and Tyr^{419} phosphorylations in the biological changes of RN1 cells after induction.

Levels of tyrosine phosphorylations of five cellular proteins in RN1 cells changed concomitant with the cell rounding (Fig. 1). These non-identified, tyrosine-phosphorylated cellular proteins are likely to mediate the morphological change, even if not all proteins are required. Identification of these proteins may also contribute to understanding of the mechanism of cell rounding in RN1 cells after induction.

Cell spreading and rounding are regulated in normal cellular activities such as cell division, cellular migration, and morphogenetic processes in epithelial cells and neurons [23]. Several lines of evidence suggest that c-Src is involved in the activities [5,7,24–26]. The spherical RN1 cells retained an anchorage dependency on cell growth. In addition, cell mixing experiments in which either 50, 500, or 5000 RN1 cells were mixed with 5×10^5 normal NIH 3T3 cells [16], resulted in lack of focus-forming ability, irrespective of IPTG treatment (data not shown). These data indicated that these cells have properties distinguishable from those of v-*src*-transformed NIH 3T3 cells, which exhibited anchorage-independent growth potential and focus-forming ability. Thus, induction of cell rounding with overexpression of human *c-src* appeared to be related to the normal cellular function of c-Src, and not to transforming activity. The cell line conditionally altered in cell morphology by regulating the expression of the c-*src* gene should be useful to reveal the molecular mechanism for cell rounding by c-Src, because the overexpressed c-Src affects cell morphology without affecting anchorage dependence of growth, in contrast with v-*src* transformation.

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